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(54) Title: INSULIN MIMETICS FROM HONEY

## (57) Abstract

The use of honey as a source material of P and A-type inositolphosphoglycans (IPGs) is disclosed, together with P and A-type cyclitol containing carbohydrates isolated from honey having the biological activities of activating PDH phosphatase or inhibiting cAMP dependent protein kinase and regulating lipogenesis respectively.

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Insulin Mimetics From HoneyField of the Invention

The present invention relates to insulin mimetics as obtainable from honey, and in particular to cyclitol containing carbohydrates as obtainable from honey which have the biological activities of P or A-type inositolphosphoglycans (IPG).

Background of the Invention

Many of the actions of growth factors on cells are thought to be mediated by a family of inositol phosphoglycan (IPG) second messengers (Rademacher et al, 1994). It is thought that the source of IPGs is a "free" form of glycosyl phosphatidylinositol (GPI) situated in cell membranes. IPGs are thought to be released by the action of phosphatidylinositol-specific phospholipases following binding of growth factors to receptors on the cell surface. There is evidence that IPGs mediate the action of a large number of growth factors including insulin, nerve growth factor, hepatocyte growth factor, insulin-like growth factor I (IGF-I), fibroblast growth factor, transforming growth factor  $\beta$ , the action of IL-2 on B-cells and T-cells, ACTH signalling of adrenocortical cells, IgE, FSH and hCG stimulation of granulosa cells, thyrotropin stimulation of thyroid cells, cell proliferation in the early developing ear and rat mammary gland.

Soluble IPG fractions have been obtained from a variety of animal tissues including rat tissues (liver, kidney, muscle, brain, adipose, heart) and bovine liver. IPG biological activity has also been detected in malaria parasitized RBC and mycobacteria. The family of IPG second messengers have been divided into distinct A and P-type subfamilies on the basis of their biological

activities. In the rat, release of the A- and P-type mediators has been shown to be tissue-specific (Kunjara et al, 1995).

5 WO98/11116 and WO98/11117 disclose the purification, isolation and characterisation of P and A-type IPGs from human tissue. Prior to these applications, it had not been possible to isolate single components from the tissue derived IPG fractions, much less in sufficient 10 quantities to allow structural characterisation.

Accordingly, while some prior art studies describe the biological activities of the IPG containing fractions, speculation as to the identity of the active components from non-human sources of the fractions were based on 15 indirect evidence from metabolic labelling and cleavage techniques.

#### Summary of the Invention

The present invention is based on the finding that honey 20 can be a source of insulin mimetics, in particular inositolphosphoglycans such as P and A-type IPGs. The results described herein show that purified fractions obtained from honey have characteristic biological properties of IPGs, such as activating pyruvate 25 dehydrogenase (PDH) phosphatase (P-type), inhibition of cAMP dependent protein kinase (P and A-types) and induction of lipogenesis in adipocytes (A-type).

While honey produced by insects such as bees from plant 30 nectar has been used in the past as a source of antibacterial agents and in the treatment of various ailments including diabetes mellitus, the present invention provides the first demonstration that IPGs are the source of the insulin mimetic activity of honey and 35 that it is possible to use honey as a natural source

material from which IPGs can be isolated and purified.

The experiments described below further show that a protein is not responsible for the insulin mimetic activity extracted from honey as it was recovered from preparations in boiled formic acid. The use of charcoal in the extraction procedure also suggests that the biological activity is not due to a nucleotide and it is unlikely to be a lipid since honey is essentially devoid of this class of compounds.

Honey is a mixture of carbohydrates, mainly monosaccharides, mixed with disaccharides and trisaccharides. Among the monosaccharides is inositol which accounts for 0.01 to 0.20% of the total sugars (Horvath et al, 1997). Therefore, the presence of inositol in honey supports the hypothesis that the insulin mimetic agents extracted could be inositolphosphoglycans, especially as they elute from anion exchange resins at pH 2.0 and 1.3, activate pyruvate dehydrogenase phosphatase, and inhibit cAMP dependent protein kinase, in addition to lowering blood glucose. The insulin mimetic agent in low doses stimulated lipogenesis from glucose, but was inhibitory at higher doses.

As discussed further below, the insulin mimetic activity was purified from honey and can be readily isolated by methods used for the extraction of IPGs from animal tissues. The biological activity of the extracts may be considered as pro-drugs for the management of the hyperglycaemia and probably the lipodystrophy of diabetes.

Accordingly, in a first aspect, the present invention

provides a substance as obtainable from honey, wherein the substance is a cyclitol containing carbohydrate which is:

- 5 (i) a P-type substance having the biological activity of activating pyruvate dehydrogenase (PDH) phosphatase; or,
- (ii) an A-type substance having the biological activity of regulating lipogenic activity and inhibiting cAMP dependent protein kinase.

10

In a further aspect, the present invention provides a P or A-type substance purified from honey.

15 As used herein, "purified" means that composition or fraction including the IPGs is free from one or more of the contaminants with which it is associated in bee honey, e.g. non-cyclitol containing carbohydrates, proteins or nucleotides. In the examples, the IPGs are purified 2000 to 7000 fold from bee honey and could be 20 further purified to isolation using the methods described in WO98/11116, WO8/11117 or as set out in Caro et al, 1997. Alternatively or additionally, purification or isolation can be carried out using cellulose in column chromatography as described below.

25

In a further aspect, the present invention provides a P or A-type substance as obtainable from honey by:

- 30 (a) making an extract by heat and acid treatment of honey;
- (b) after centrifugation and charcoal treatment, allowing the resulting solution to interact overnight with an AG1-X8 (formate form) anion exchange resin;
- 35 (c) collecting a fraction having P-type substance activity obtained by eluting the column with 10 mM HCl and/or collecting a fraction having A-type substance

activity obtained by eluting the column with 50 mM HCl;

(d) neutralising to pH 4 (not to exceed pH 7.8) and lyophilising the fractions to isolate the substances;

(e) employing descending paper chromatography using  
5 4/1/1 butanol/ethanol/water as solvent;

(f) purification using high-voltage paper electrophoresis in pyridine/acetic acid/water; and,

(g) purification and isolation using Vydac HPLC chromatography to obtain the isolated P or A-type  
10 substance.

In a further aspect, the present invention provides the use of honey as a source material for the purification or isolation of P or A-type substances.

15 In a further aspect, the present invention provides the use of cellulose column chromatography in the purification of P or A-type substances.

20 In a further aspect, the present invention provides a method of purifying or isolating IPGs, such as the P or A-type substances disclosed herein, the method comprising contacting a sample containing IPGs with a column containing cellulose, and eluting the IPGs from the  
25 column.

30 Preferably, the method employs microcrystalline cellulose. Conveniently, the IPG-containing fraction is dissolved 4/1/1 butanol/water/ethanol (B:W:E) and loaded onto the column. The column is preferably washed with B:W:E and methanol, and then eluted with water and HCl and the eluates collected. The eluates can then be concentrated and freeze dried. In a preferred embodiment, the cellulose purification corresponds to the  
35 conditions used in the examples.

In a further aspect, the present invention provides a pharmaceutical composition comprising P and/or A-type substance, or purified fraction comprising a P and/or A-type substance, as obtainable from honey, in combination 5 with a carrier.

In a further aspect, the present invention provides the use of a P or A-type substance as obtainable from honey for the preparation of a medicament for the treatment of 10 a condition characterised by a deficiency or imbalance of the IPGs. Examples of conditions mediated by IPGs, e.g. as growth factor second messengers, are set out in more detail below and includes the treatment of diabetes.

15 Embodiments of the present invention will now be described by way of example in more detail below.

#### Detailed Description

##### IPGs and IPG Analogues

20 Studies have shown that A-type mediators modulate the activity of a number of insulin-dependent enzymes such as cAMP dependent protein kinase (inhibits), adenylyl cyclase (inhibits) and cAMP phospho-diesterases (stimulates). In contrast, P-type mediators modulate the 25 activity of insulin-dependent enzymes such as pyruvate dehydrogenase phosphatase (stimulates), glycogen synthase phosphatase (stimulates) and cAMP dependent protein kinase (inhibits). The A-type mediators mimic the lipogenic activity of insulin on adipocytes, whereas the 30 P-type mediators mimic the glycogenic activity of insulin on muscle. Both A-and P-type mediators inhibit cAMP dependent protein kinase and are mitogenic when added to fibroblasts in serum free media. The ability of the mediators to stimulate fibroblast proliferation is 35 enhanced if the cells are transfected with the EGF-

receptor. A-type mediators can stimulate cell proliferation in the chick cochleovestibular ganglia.

Soluble IPG fractions having A-type and P-type activity  
5 have been obtained from a variety of animal tissues including rat tissues (liver, kidney, muscle brain, adipose, heart) and bovine liver. A- and P-type IPG biological activity has also been detected in human liver and placenta, malaria parasitized RBC and mycobacteria.  
10 The ability of an anti-inositolglycan antibody to inhibit insulin action on human placental cytotrophoblasts and BC3H1 myocytes or bovine-derived IPG action on rat diaphragm and chick cochleovestibular ganglia suggests cross-species conservation of many structural features.

15 A-type substances are cyclitol-containing carbohydrates, also containing  $Zn^{2+}$  ion and optionally phosphate and having the properties of regulating lipogenic activity and inhibiting cAMP dependent protein kinase. They may  
20 also inhibit adenylyl cyclase, be mitogenic when added to EGF-transfected fibroblasts in serum free medium, and stimulate lipogenesis in adipocytes.

25 P-type substances are cyclitol-containing carbohydrates, also containing  $Mn^{2+}$  and/or  $Zn^{2+}$  ions and optionally phosphate and having the properties of regulating glycogen metabolism and activating pyruvate dehydrogenase phosphatase. They may also stimulate the activity of glycogen synthase phosphatase, be mitogenic when added to  
30 fibroblasts in serum free medium, and stimulate pyruvate dehydrogenase phosphatase.

Methods for obtaining A-type and P-type IPGs are set out in Caro et al, 1997, and in WO98/11116 and WO98/11117.  
35 In summary, the methods disclosed in these applications

involve:

- (a) making an extract by heat and acid treatment of IPG source material;
  - 5 (b) after centrifugation and charcoal treatment, allowing the resulting solution to interact overnight with an AG1-X8 (formate form) anion exchange resin;
  - 10 (c) collecting a fraction having P-type IPG activity obtained by eluting the column with 10 mM HCl and/or collecting a fraction having A-type IPG activity obtained by eluting the column with 50 mM HCl;
  - (d) neutralising to pH 4 (not to exceed pH 7.8) and lyophilising the fractions to isolate the substances;
  - 15 (e) employing descending paper chromatography using 4/1/1 butanol/ethanol/water as solvent;
  - (f) purification using high-voltage paper electrophoresis in pyridine/acetic acid/water; and,
  - (g) purification and isolation using Vydac HPLC chromatography to obtain the isolated IPGs.
- 20 As disclosed herein, it is also possible to employ column chromatography using cellulose, and especially microcrystalline cellulose, in the isolation or purification of IPGs. Exemplary conditions are provided in the experimental section below.

25

#### Pharmaceutical Compositions

The mediators and analogues of the invention can be formulated in pharmaceutical compositions. These compositions may comprise, in addition to one or more of the mediators, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of

administration, e.g. oral, intravenous, cutaneous or subcutaneous, nasal, intramuscular, intraperitoneal routes.

5 Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal  
10 or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

15 For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of  
20 relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as sodium chloride injection, Ringer's injection, lactated Ringer's injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be  
25 included, as required.

30 Preferably, the pharmaceutically useful compound according to the present invention is given to an individual in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual.  
35 Typically, this will be to cause a therapeutically useful effect, e.g. in the treatment of diabetes. The actual amount of the compounds administered, and rate and time-

course of administration, will depend on the nature and severity of the condition being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 16th edition, Osol, A. (ed), 1980.

The compositions disclosed herein can be used in the treatment of conditions involving a deficiency of a P or A-type substance and conditions in which the ratio of P and A-type substances is imbalanced, e.g. by administering one of the substance or an antagonist to correct the ratio of the substances.

By way of example, the compositions of the invention can be used in the treatment of diabetes, including diabetes due to insulin resistance, insulin resistance in type I diabetes and brittle diabetes. The compositions may also be used to treat other conditions mediated by insulin, and in particular insulin resistance or insulin underproduction such as lipotrophic disorders or polycystic ovary disease.

The role and uses of P and A-type IPGs in the diagnosis and treatment of diabetes is disclosed in WO98/11435. In summary, this application discloses that in some forms of diabetes the ratio of P:A type IPGs is imbalanced and can be corrected by administering a medicament comprising the appropriate ratio of P or A-type IPGs or antagonists thereof. In particular, the application describes the

treatment of obese type II diabetes (NIDDM) patients with a P-type IPG and/or an A-type IPG antagonist and the treatment of IDDM (type I diabetes) or lean type II diabetes (NIDDM) (body mass index < 27) with a mixture of 5 P and A-type IPGs, typically in a P:A ratio of about 6:1 for males and 4:1 for females. The IPGs isolated or purified from honey as described herein can be employed in the treatment of diabetes described in the earlier application. Similarly, the IPGs from honey disclosed 10 herein could be used in the treatment of other conditions characterised by a deficiency or imbalance of P and/or A-type IPGs.

#### Materials and Methods

##### 15 Isolation of insulin mimetic activity from honey

500 g resin of AG1X8 resin (BioRad) was suspended in deionized water and allowed to settle for 5 minutes. The water and fines were decanted. The fining process was repeated until the washings were clear. The resin was 20 suspended in 3840 ml water and 160 ml concentrated formic acid was added. The pH of the supernatant was less than pH 2.0 and the molarity was 1.06 M. The resin was then left overnight at room temperature. The resin was then washed with water until the pH of the washing was 4.8 and 25 was stored in water at 4°C.

30 Acid washed charcoal was washed in deionized water to remove fines and filtered using a Buchner funnel and flask. The fined charcoal was then dried in air at room temperature.

1 ml concentrated formic acid was added to 499 ml deionized water. 190.1 mg Na<sub>2</sub>EDTA was added to the solution and stirred to dissolve. This produced a 35 solution in which the molarity of formic acid was 0.053

M. Immediately before use, 50 µl 2-mercaptoethanol was added.

5        5 ml honey (Gale's) was mixed with 50 ml of the formic acid:mercaptoethanol:EDTA solution (50:1:1 mmol/l) and heated to boiling for 5 minutes with shaking. The solution was immediately cooled on ice and 1 g of the acid-washed charcoal added. The mixture was stirred magnetically in the cold room for 30 minutes. Next, the  
10      mixture was centrifuged at 40,000 × g for 30 minutes at 4°C. The pH of the supernatant was adjusted to pH 6.0 with 10% NH<sub>4</sub>OH, and centrifuged again at 40,000 × g for 30 minutes at 4°C. Where traces of charcoal remained in the supernatant, it was filtered through a 0.4 µm cellulose  
15      acetate filter cartridge using a syringe.

The supernatant was mixed with 25 ml of settled BioRad AG1X8 (formate) pH 4.5 and gently mixed on an orbital shaker for 20 hours at 4°C. The resin was transferred to  
20      a column and washed with 5 bed-volumes (125ml) water, and then with 5 bed-volumes (125 ml) of 1 mmol/l HCl pH 3.0. One fraction containing the insulin mimetic activity was then eluted with 10 bed-volumes (250 ml) of 10 mmol/l HCl (pH 2.0). A second fraction containing an insulin  
25      mimetic activity was eluted with 10 bed-volumes (250 ml) of 50 mmol/l HCl (pH 1.3). The pH of the eluates was then adjusted to 4.0 with 10% NH<sub>4</sub>OH, and then were concentrated under reduced pressure in a rotary evaporator at 37°C. The concentrated eluates were then  
30      lyophilised. The pH 2.0 fraction was further purified using the pH 2.0 fraction clean-up procedure described below.

**Clean-up procedure for pH 2.0 fraction**

35      50 g of cellulose powder (microcrystalline cellulose for

column chromatography available from Merck) was suspended in 1000 ml deionized water and allowed to sediment for 5 minutes. The suspended fines were decanted and this was repeated 5 times. The settled cellulose was then 5 suspended in 50% ethanol and stored at 4°C.

The prepared cellulose was suspended in 50% ethanol/water and about 2 ml was transferred to each column and allowed to settle. The settled cellulose was washed with 5 ml 10 deionized H<sub>2</sub>O and the liquid level was allowed to drain to the top of the cellulose. The column was then washed with 5 ml butanol:water:ethanol (B:W:E) (4:1:1) and the liquid level allowed to drain to the top of the column. The columns were then capped and stored at 4°C prior to 15 use.

The freeze-dried pH 2.0 fraction prepared above was dissolved in 500 µl B:W:E (4:1:1), and the solution loaded onto the column and allowed to drain into the top 20 of the cellulose. The column was washed with 5 ml B:W:E (4:1:1) and 5 ml methanol, and the washings discarded. The column was then eluted with (a) 5 ml deionized water and the eluate collected (W) and (b) 10 ml of 50 mM HCl (pH 1.3) and the eluate collected (A). The eluates were 25 then concentrated in a Vapour-Mix concentrator and then freeze dried.

#### **PDH phosphatase assay**

The following reagents and protocol were used in assays 30 for PDH phosphatase activity. All reagents were stored at -80°C until use.

**Mixture A:** 1 part coenzyme A (60 mg +60 ml 3.2 mM DTT), 1 part dithiothreitol (DTT) (3.2 mM), 1 part NAD (25 mM), 1 35 part dipotassium hydrogen phosphate (KPi) (500 mM/pH

8.0), 1 part thiamine pyrophosphate.HCl (TPP) (2 mM) and 5 parts water. Mixture A was stored in 10 ml aliquots.

5 **Mixture B:** 1 part ATP (10 mM), 1 part DTT (10 mM), 1 part ethylene glycol tetraacetic acid (EGTA) (1 mM), 1 part MgCl<sub>2</sub>(10 mM), 1 part KPi (200 mM/pH 7.0), 3 parts water. Mixture B was stored in 0.4 ml aliquots.

10 **Mixture C:** 4 parts BSA (1 mg/ml), 1 part CaCl<sub>2</sub>(1 mM), 1 part DTT (10 mM), 1 part MgCl<sub>2</sub> (100 mM), 1 part KPi 200 mM/pH 7.0. Mixture C was stored in 2 ml aliquots.

**NaF:** 780 mM NaF was stored in 1.5 ml aliquots.

15 **Pyruvate:** 40 mM pyruvate (PYR) was stored in 0.5 ml aliquots.

20 PDH (100 µl) was added to a tube containing reaction mixture B and was mixed and incubated at 30°C for 15 minutes (or until PDH activity was 99% inactivated). The mixture was stored in ice. A 50 µl aliquot of this mixture was transferred to each cuvette for the assay. Mixture C (200 µl) was then added and mixed well, and the resulting mixture incubated at 30°C for 3 minutes. The 25 test solution (10-20 µl) was then added, followed by PDH phosphatase (10 µl), and the resulting solution mixed well and incubated at 30°C for 3 minutes. Next, 50 µl NaF was added followed by mixture A (1000 µl) with mixing. The PDH assay was started by the addition of 50 µl pyruvate, and the absorbance at 340 nm was recorded 30 for at least 5 minutes.

#### **Protein kinase A inhibition assay**

The following reagents and protocol were used in assays

for protein kinase A inhibition.

**Reaction buffer:** ATP (10 mM), MgCl<sub>2</sub> (50 mM), 0.01% Triton X-100, Tris (100 mM/pH 7.4).

5

**Activator solution:** 500 µM cAMP in water.

**PKA substrate:** 1.2 mg Kemptide (LRRASLG) labelled with a fluorescent probe in 550 µl 1% BSA.

10

**Phosphopeptide binding buffer:** NaAc (0.1 M), NaCl (0.5 M, 0.2% NaN<sub>3</sub>, pH 5.0).

15

**Phosphopeptide elution buffer:** NH<sub>4</sub>HCO<sub>3</sub> (0.1 M), 0.02% NaN<sub>3</sub>, pH 8.0.

Reaction buffer, PKA substrate and activator solution (5 µl each) were premixed for each test sample. Duplicates of the following solutions were pipetted into the tubes:

20

Reagent	Test	+ve control	-ve control
Reaction Mixture	15	15	15
PKA dilution buffer	-	5	10
PKA standard (200U/ml)	5	5	-
Unknown (in PKA diluent)	5	-	-

25

The tubes were capped and the contents mixed vigorously and incubated for 30 minutes at 30°C. A 20 µl sample from each tubes was then transferred to the affinity membrane of an individual separation unit. Binding buffer (250 µl) was then applied to each unit and incubated for 3 minutes at room temperature. The units were then centrifuged at 6500 rpm for 1 minute.

A further aliquot of binding buffer (250 µl) was applied to the unit, followed by incubation for 3 minutes, and centrifugation at 6500 rpm for 1 minute. The binding buffer collected contained the non-phosphorylated Kemptide. Elution buffer (250 µl) was applied to each unit and was incubated for 3 minutes at room temperature, and then centrifuged at 6500 rpm for 1 minute. This step was repeated. The collected binding buffer contains the phosphorylated Kemptide.

10

The eluate (250-300 µl) was transferred to individual wells of a flat-bottom 96-well plate and the absorbance at 570 nm read using a spectrophotometer or a fluorometer (with excitation at 573 nm and emission at 589 nm).

15

#### **Lipogenesis assay**

This was carried out on adipocytes isolated from the epididymal fat pads of young rats, measuring the rate of incorporation of uniformly labelled glucose into fatty acids as a measure of lipogenesis.

Epididymal fat pads from three rats were dissected out and chopped finely with scissors in a plastic weighing boat containing some Krebs Ringer Hepes Albumin buffer (KRHA). The KRHA was decanted and the adipose tissue suspended in fresh KRHA (15 ml) containing 30 mg collagenase-D and 2% BSA (fatty acid free). The suspension was incubated in a water bath at 37°C with shaking, until the end point of the digestion (45-50 minutes). The fat fragments were aspirated several times to obtain a homogenous suspension, the suspension strained, diluted with KRHA to 30 ml and centrifuged at room temperature. The supernatant was withdrawn and replaced with fresh KRHA (30 ml) and the adipocytes resuspended by gentle mixing. The centrifugation steps

were repeated twice more. The adipocytes were then suspended in 30 ml KRHA in a shaking water bath with gassing ( $O_2/CO_2$  95:5)

5 For each assay, a vial was loaded with 500  $\mu$ l substrate (KRHA and  $U^{14}C$ -glucose) and the test solution or 1 nM insulin as positive control. Samples of the adipocyte suspension (0.5 ml), and an eppendorf tube were inserted, the vial sealed and gassed for 10 minutes. Gassing was  
10 then stopped and the suspension incubated for 2 hours. At 0.5 minutes intervals, hyamine hydroxide (0.2 ml) was injected into the eppendorf tube followed by HCl (0.2 ml/6N) into the incubation mixture. The vial was allowed to equilibrate for 60 minutes, after which the hyamine  
15 hydroxide was transferred to a clean vial using methanol. Scintillation cocktail (4 ml) was added and  $^{14}CO_2$  was counted.

The vial contents were transferred to a tube using  
20 isopropanol/hexane/ $H_2SO_4$  (40/10/1). Hexane and water were added with vigorous mixing and the phases allowed to separate. An aliquot of  $H_2SO_4$  (0.001 N) was added. The phases were then separated by centrifugation at 500  $\times g$  for 10 minutes at 4°C. The hexane phase was then  
25 transferred to a pico vial, allowed to dry in a fume hood. The resulting lipid was dissolved in 4 ml non-aqueous scintillation mixture, and the radioactivity incorporated into lipids was counted.

30 **Induction of streptozotocin diabetes in Olac rats**

0.05 M citrate buffer pH 4.5 was prepared by dissolving 1.05 g citric acid monohydrate in 80 ml 0.154 M NaCl and adjust pH to 4.5 with 1.0 M NaOH. The volume was made up to 100 ml with 0.154 M NaCl. Streptozotocin (STZ) was  
35 prepared by dissolving 100 mg streptozotocin in 1.6 ml

0.05 M citrate buffer pH 4.5 in 0.154 M NaCl. Each rat was injected with 0.2 ml of STZ i.v. through the tail vein. Rats were tested for hyperglycaemia and used one week later. pH 1.3 or 2.0 fractions were injected i.v.

5 in the doses indicated below in 0.20 ml PBS (Fonteles et al, 1996).

### Results

#### **Purification of insulin mimetic compounds**

10 Recovery of insulin mimetic activity from cellulose columns:

Original activity	61.2 U/ml
Water + Acid eluates	32.2 U/ml
Recovery	52.5%

15

This result shows that cellulose chromatography can be used in the purification of IPGs from natural source materials.

20 **PDH phosphatase assay**

PDH phosphatase stimulating activity:

24 April 98	61.2 U/ml
6 June 98	53.0 U/ml
9 June 98	112.0 U/ml
25 27 July 98	84.9 U/ml
Mean activity	73.6 U/ml ± 12.4 (SE)

#### **Protein kinase A inhibition assay**

The pH 2.0 and 1.3 fractions from 2.5 ml honey were each dissolved in 50 µl water. 5 µl (equivalent to 0.25 ml honey) was assayed for PKA inhibitory activity in duplicate.

35 pH 2.0 fraction                    72% inhibition equivalent to 2.9  
    PKA units/ml

pH 1.3 fraction                    70% inhibition equivalent to 2.8  
PKA units/ml

5        The results show that honey contains both A and P-type  
activities in comparable amounts.

#### Glucose oxidation assay

	$^{14}\text{CO}_2$ (cpm) $\pm$ SE	Percent activation $\pm$ SE
	5 mM glucose	1577 $\pm$ 163
10	0.05 ml honey	113 $\pm$ 11
	0.10 ml honey	111 $\pm$ 6
	0.20 ml honey	104 $\pm$ 4
	0.40 ml honey	94 $\pm$ 11
	0.50 ml honey	95 $\pm$ 8

15        Linear Regression:  $y = -44x + 114.4$ ,  $R^2 = 0.9392$

#### Lipogenesis assay

	$^{14}\text{C-Lipids}$ (cpm) $\pm$ SE	Percent activation $\pm$ SE
	5 mM glucose	1061 $\pm$ 25
20	0.05 ml honey	108 $\pm$ 4
	0.10 ml honey	101 $\pm$ 5
	0.20 ml honey	87 $\pm$ 2
25	0.40 ml honey	78 $\pm$ 5
	0.50 ml honey	73 $\pm$ 3

Linear Regression:  $y = -74.333x + 107.98$ ,  $R^2 = 0.9363$

30        The A-type stimulated glucose oxidation and lipogenesis in adipocytes in a dose-dependent manner. However, as high doses, there was an inhibition of lipogenesis implying that there may be more than one component in the fraction.

**Effect of IPGs from honey on blood glucose of  
streptozotocin diabetic rats**

5	Fraction	ml eq.	Initial Blood Glucose	Blood Glucose % Change from Initial				
				0h	1h	2h	3h	4h
		0	7.7±0.6	100±7.8	87±14	101±1	101±8	97±11
	pH2.0	1	24.9±0.8	100±3.2	78±3	72±14	78±14	75±21
		2	30.4±0.6	100±2	101	76	71	66
		3	21.8±1	100±4.6	90	82	95	82
10	pH1.3	1	23±0.4	100±1.7	113±2	103±4	95±1	95±10
		2	28.9±2.6	100±9	93±4	91±5	80±3	68±2
		3	19±0	100±0	100±11	90±9	85±2	85±2

Both the pH 2.0 (P-type) and the pH 1.3 (A-type)  
15 fractions induce lowering of blood glucose in  
streptozotocin diabetic rats. The effect lasts for up to  
four hours and is dose-dependent.

References:

The references mentioned herein are all expressly incorporated by reference.

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15 Lilley et al, Arch. Biochem. Biophys., 296:170-174, 1992.

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Claims:

1. A substance as obtainable from honey, wherein the substance is a cyclitol containing carbohydrate which is:

- 5 (i) a P-type substance having the biological activity of activating pyruvate dehydrogenase (PDH) phosphatase; or,
- (ii) an A-type substance having the biological activity of regulating lipogenic activity and inhibiting cAMP dependent protein kinase.

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2. The substance of claim 1 as obtainable from honey by:

(a) making an extract by heat and acid treatment of honey;

15 (b) after centrifugation and charcoal treatment, allowing the resulting solution to interact overnight with an AG1-X8 (formate form) anion exchange resin;

(c) collecting a fraction having A-type substance activity obtained by eluting the column with 50mM HCl (pH 1.3) and/or collecting a fraction having P-type substance activity obtained by eluting the column with 10mM HCl (pH 2.0);

(d) neutralising to pH 4 (not to exceed pH 7.8) and lyophilising the fraction to isolate the substance;

20 (e) employing descending paper chromatography using 4/1/1 butanol/ethanol/water as solvent;

(f) purification using high voltage paper electrophoresis in pyridine/acetic acid/water; and,

25 (g) purification and isolation using Vyda<sup>®</sup> HPLC chromatography to obtain the isolated P or A-type substance.

3. The substance of claim 1 or claim 2 for use in a method of medical treatment.

4. Use of honey as a source material for a P or A-type substance, wherein the substance is a cyclitol containing carbohydrate which is:

- 5 (i) a P-type substance having the biological activity of activating pyruvate dehydrogenase (PDH) phosphatase; or,
- (ii) an A-type substance having the biological activity of regulating lipogenic activity and inhibiting cAMP dependent protein kinase.

10

5. A pharmaceutical composition comprising one or more of the substances of claim 1 or claim 2.

15

6. Use of a substance of claim 1 or claim 2 for the preparation of a medicament for the treatment of a condition characterised by a deficiency or imbalance of IPGs.

20

7. The use of claim 6, wherein the condition is diabetes.

8. The use of claim 7, wherein the diabetes is diabetes due to insulin resistance (Type II diabetes), Type I diabetes, or brittle diabetes.

25

9. The use of claim 6, wherein the condition is a lipotrophic disorder.

30

10. The use of claim 6, wherein the condition is polycystic ovary disease.

11. Use of cellulose chromatography for purifying or isolating a P or A-type substance, wherein the substance is a cyclitol containing carbohydrate which is:

35

- (i) a P-type substance having the biological

activity of activating pyruvate dehydrogenase (PDH) phosphatase; or,

(ii) an A-type substance having the biological activity of inhibiting cAMP dependent protein kinase.

5

12. The use of claim 11, wherein the use involves contacting a sample containing P or A-type substance with a column containing cellulose and eluting the substance from the column.

10

13. The use of claim 11 or claim 12, wherein the column comprises microcrystalline cellulose.

15

14. A method of purifying or isolating a P or A-type substance, wherein the substance is a cyclitol containing carbohydrate which is:

(i) a P-type substance having the biological activity of activating pyruvate dehydrogenase (PDH) phosphatase; or,

(ii) an A-type substance having the biological activity of inhibiting cAMP dependent protein kinase;

wherein the method comprises:

(a) loading a column containing cellulose with a sample containing the P or A-type substance so that P or A-type substance binds to the column; and,

(b) eluting the P or A-type substance from the column.

25

15. The method of claim 14, wherein the cellulose is microcrystalline cellulose.

30

16. The method of claim 14 or claim 15 further comprising the step of dissolving the sample containing the P or A-type substance in 4/1/1 butanol/water/ethanol (B:W:E) prior loading on the column.

35

17. The method of any one of claims 14 to 16, further comprising the step of washing the column with B:W:E and methanol.

5 18. The method of any one of claims 14 to 17, wherein the P or A-type substance is eluted with water and HCl.